

Inhibition of multidrug efflux as a strategy to prevent biofilm formation

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1 **Co-ordinated regulation of multidrug efflux and biofilm formation in**
2 ***Salmonella*.**

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12

13 **Abstract**

14 **Objectives.** We have recently shown that inactivation of any of the multidrug efflux
15 systems of *Salmonella* results in loss of ability to form a competent biofilm, the aim of
16 this study was to determine the mechanism linking multidrug efflux and biofilm
17 formation.

18 **Methods.** Mutants lacking components of the major AcrAB-TolC system were
19 investigated for their ability to form a biofilm, aggregate and produce biofilm matrix
20 components. The potential for export of a biofilm relevant substrate via efflux pumps
21 was investigated as well as expression of genes that regulate multidrug efflux and
22 production of biofilm matrix components.

23 **Results.** Mutants of *Salmonella enterica* serovar Typhimurium which lack TolC or
24 AcrB but surprisingly not AcrA were compromised in their ability to form biofilms.
25 This defect was not related to changes in cellular hydrophobicity, aggregative ability
26 or export of any biofilm specific factor. The biofilm defect associated with inactivation
27 of *acrB* or *tolC* resulted from transcriptional repression of curli biosynthesis genes
28 and consequent inhibition of the production of curli by mutants lacking AcrB or TolC.
29 This repression was associated with up-regulation of the global regulator, *ramA* and
30 artificial over-expression of *ramA*, *marA* and *soxS* each decreased biosynthesis of
31 curli, and inhibited biofilm formation. However, inactivation of these regulators did not
32 rescue the ability of efflux mutants to form a biofilm.

33 **Conclusions.** This work shows biofilm formation and multidrug efflux are co-
34 ordinally regulated, and that transcriptional repression of curli biosynthesis causes
35 a lack of biofilm formation which occurs in response to lack of efflux activity or as a
36 result of over-expression of global regulators *ramA*, *marA* and *soxS*.

37

38 **Introduction**

39 Bacterial biofilms are a major clinical and industrial problem and eradication of
40 biofilms presents a challenge for antimicrobial chemotherapy.¹⁻³ Bacteria within a
41 biofilm are encased within an extracellular matrix which commonly includes
42 polysaccharides, proteins and other species specific components.^{1,4} Multidrug
43 resistance efflux (MDR) pumps are transporters which can export a wide range of
44 xenobiotics including antibiotics, dyes, biocides and other toxic molecules preventing
45 lethal accumulation within the cell.⁵ The expression of efflux pumps is tightly
46 regulated and efflux genes are usually subject to control by both local and global
47 regulators.⁵ *Salmonella enterica* serovar Typhimurium (*S. Typhimurium* hereafter)
48 has nine MDR systems from four separate protein families; the major system in
49 *Salmonella* is the AcrAB-TolC RND system.⁶ The homologous global transcription
50 regulators MarA, RamA and SoxS can all increase expression of *acrAB* in response
51 to stress.⁷⁻⁸ We have recently described an inability to form a competent biofilm
52 associated with inactivation of any of the MDR systems of *S. Typhimurium* including
53 those which are normally cryptic in standard laboratory conditions.⁹ We found that
54 production of curli, a major component of the *Salmonella* biofilm extracellular matrix
55 was defective in all these strains, suggesting a common mechanism for the lack of
56 biofilm formation in all mutants.

57 Here, using AcrAB-TolC as a paradigm we investigated the mechanism by which
58 loss of efflux activity results in a lack of curli production. We ruled out export of a
59 factor crucial for biofilm development via AcrAB-TolC and also showed that
60 inactivation of components of AcrAB-TolC did not alter cellular hydrophobicity.
61 However, inactivation of efflux components was found to significantly alter
62 expression of biofilm related genes. We demonstrate that the biofilm defect of

63 mutants lacking AcrB or TolC is due to transcriptional repression of curli biosynthesis
64 in the efflux mutants. Additionally, over-expression of the global regulators *ramA*,
65 *marA* or *soxS* resulted in repression of curli biosynthesis and loss of biofilm
66 formation. This work demonstrates a mechanism whereby loss of MDR efflux pumps
67 impacts production of a biofilm due to co-ordinated regulation of efflux and biofilm
68 formation.

69

70 **Materials and methods**

71 ***Strains and growth media***

72 All strains used in this study and their origins are shown in Table 1. *S. Typhimurium*
73 ATCC 14028S (L828) was used as a control strain throughout. Isogenic derivatives,
74 L829 (*tolC::cat*) and L830 (*acrB::aph*) have been described previously,⁶ New
75 mutants were created by transduction of mutant alleles into L828, and resistance
76 marker cassettes were removed by plasmid pCP20 as previously described.¹⁰ To
77 select for transductants, 100 µl from each transduction reaction was spread onto LB
78 plates supplemented with 50 mg/L of kanamycin or 25 mg/L of chloramphenicol and
79 incubated overnight at 37°C. Transfer of each mutant allele was verified by PCR and
80 sequencing. Strains were stored at -20°C on Protect™ beads and routinely cultured
81 on Luria-Bertani agar or broth unless stated otherwise. Over-expression and
82 complementation plasmids containing *marA*, *soxS* or *ramA* were constructed in
83 pTRC and pWKS30 as previously described.⁷

84 ***Biofilm formation assays***

85 Various models were used to analyse biofilm formation in this study.
86 For crystal violet biofilm assays, overnight cultures of strains were diluted in fresh
87 Luria-Bertani broth without salt to an optical density of 0.1 at 600 nm. 96 well
88 polystyrene microtitre trays (Sterilin) were inoculated with 200 µl of this suspension
89 and incubated at 30°C for 48 hours with gentle agitation. After incubation liquid was
90 removed from all wells and wells were washed with sterile distilled water to remove
91 any unbound cells. Biofilms were stained by adding 200 µl of 1% crystal violet to
92 appropriate wells for 15 minutes. Crystal violet was removed and each well washed
93 with sterile distilled water to remove unbound dye. The stained biofilm was
94 solubilised by adding 200 µl of 70% ethanol and optical density measured at 600 nm

95 using a FLUOstar Optima (BMG labtech). All biofilm assays were repeated three
96 times with two biological and four technical replicates per repeat.

97 To determine whether biofilm formation in L829 (*toIC::cat*) and L830 (*acrB::aph*)
98 could be rescued by co-incubation with L828 (wild-type), strains were grown
99 separated by a 0.45 µm membrane and biofilms formed as in the crystal violet assay
100 but in 500 µl volumes in 24 well transwell plates. Assays were repeated with and
101 without the presence of L828 (wild-type) in the upper 'insert' chamber with liquid
102 contiguous between the upper and lower chambers. Biofilms were stained with
103 crystal violet and quantified as above. Assays were repeated with addition of either a
104 mid-logarithmic or stationary phase culture of L828 (wild-type) to assess whether
105 growth phase had an impact upon production of any soluble biofilm promoting factor.
106 Biofilm formation under flow conditions were formed and visualised using a Bioflux
107 microfluidic system (Fluxion) and phase contrast microscopy. Flow channels were
108 inoculated with overnight cultures diluted in LB broth without salt to an optical density
109 of 0.8 at 600 nm, plates were then incubated at 30°C for three hours to allow the
110 bacteria to adhere to the flow channels. Fresh LB broth without salt was then applied
111 to the inlet wells of the plate and pumped through the flow cells at a force of 0.3
112 dynes at 30°C for 48 hours. Phase contrast microscopy was used to visualise the
113 biofilms formed at x10m, x20 and x40 magnification.

114 **Aggregation assays**

115 To examine whether loss of *acrB* or *toIC* led to alteration in cellular hydrophobicity or
116 aggregative ability two different assays were used.

117 To measure the time taken for strains to settle, strains were incubated overnight in
118 10 ml LB (without salt) broths with shaking (150 rpm) before being placed statically
119 on the bench. Samples (100 µl) were taken periodically from immediately below the

120 surface of the liquid and the optical density at 600 nm measured and recorded.
121 Enteroaggregative *E. coli* O42 was used as a positive control.
122 To determine whether there were any intrinsic differences in aggregative ability of
123 each strain ammonium sulphate was used to induce aggregation of bacterial cells, a
124 4 M stock of $(\text{NH}_4)_2\text{SO}_4$ was made in 1 X PBS and adjusted to a pH 6.8. This stock
125 was then serially diluted and mixed 1:1 (in 100 μl final volume) with bacterial
126 suspensions (adjusted from an overnight culture to an OD 570 nm of 0.8) for each
127 strain. These suspensions were immediately added to a microscope slide and
128 rocked gently for 30 min before aggregation scored visually under a microscope as
129 the presence of a precipitate. The lowest concentration of $(\text{NH}_4)_2\text{SO}_4$ required to
130 induce aggregation was recorded for each strain.

131 ***Artificial over-expression of ramA, soxS and marA***

132 pTrc-*ramA* carrying an IPTG inducible *ramA* was transformed into L828 (wild-type) to
133 observe the phenotypic effects of over expressing *ramA*.¹¹ pTrc-*soxS* and pTrc-*marA*
134 were constructed in a similar manner and introduced into L828 (wild-type), L829
135 (*tolC::cat*) and L830 (*acrB::aph*). Plasmid DNA was harvested from 10 ml cultures of
136 strains containing plasmids after overnight incubation in LB broth at 37°C using the
137 QIAprep® Spin Cell Mini Kit (QIAGEN, U.K). The resulting plasmid DNA was
138 analysed by agarose gel electrophoresis and quantified using Gene Tools software
139 (Syngene, Cambridge, U.K). Plasmid DNA was transferred into recipient cells by
140 electroporation. The impact of over-expression of each of the regulators on biofilm
141 formation was investigated in the crystal violet assay; wells containing plasmids were
142 supplemented with 100 mg/L of ampicillin and 1 mM IPTG to induce gene
143 expression.

144 ***Determination of gene expression***

145 The temporal and spatial expression of *ramA*, *marA* and *soxS* within biofilms was
146 visualised using promoter-*gfp* fusion reporter constructs in pMW82.¹² Strains
147 carrying reporter plasmids were grown overnight in LB containing 100 mg/L of
148 ampicillin then adjusted in PBS to an optical density of 0.1 at 600nm. Spots (5 μ l)
149 were inoculated onto LB – NaCl agar plates containing 100 mg/L of ampicillin and 40
150 mg/L of Congo red and incubated statically at 30 °C. Fluorescence of colonies was
151 visualised after 24 and 48 h using a Nikon SMZ800 microscope (with Integilight C-
152 HGFI fluorescence module attachment) and representative images captured. The
153 expression of each regulator gene in response to addition of EIs was also inferred
154 from measurements of fluorescence (Ex 487, Em 507) using a FLUOstar OPTIMA
155 (BMG Labtech, U.K). Fluorescence was measured in a wild-type strain every 10 min
156 over a 5 h period after the addition of a range of concentrations of the three EIs;
157 PA β N, CCCP and chlorpromazine. Strains were grown in 100 μ l of LB broth
158 (inoculated with $\sim 10^7$ cfu/ml) at 30°C with shaking throughout the experiment.
159 Induction of expression of each gene was calculated as the ration of average
160 expression (based on 8 biological replicates) of induced samples compared to un-
161 induced controls. The students 't' test was used to determine significance of
162 differences in *ramA* expression.

163 The expression of *marA*, *ramA*, *soxS*, *rob* and 16S rRNA were also determined by
164 reverse-transcriptase PCR as previously described.⁷ The expression of *csgBAC* and
165 *csgDEFG* were all determined using comparative RT-PCR, again as previously
166 described.⁹ All primers used in this study are shown in Table 2.

167 ***Staining of curli fimbriae***

168 Phenotypic differences in curli production were visualised by growing strains on agar
169 containing Congo red (40 mg/L, Sigma-Aldrich Ltd., UK) and incubating them for 48h
170 at 30° C as described previously.⁹

171

172 **Results**

173 ***Mutants lacking a functional *acrB* or *tolC* do not form competent biofilms***
174 ***whereas a mutant lacking *acrA* is able to biofilm***

175 A high throughput biofilm assay using crystal violet to stain cells adhered to a 96 well
176 plate showed a significant decrease in the biofilm formation ability of L829 (*tolC::cat*)
177 and L830 (*acrB::aph*) (figure 1). However, genetic inactivation of *acrA* (L1271
178 (*acrA::aph*)), the periplasmic adapter protein, had no negative effect on biofilm
179 formation. The phase contrast microscopy images from biofilms formed in a flow cell
180 under shear stress showed a similar pattern as the crystal violet assay with L828
181 (wild-type) forming a mature biofilm and L829 (*tolC::cat*) and L830 (*acrB::aph*)
182 adhering as individual cells to the flow cell but unable to form a mature, three
183 dimensional biofilm (figure 2).

184 ***Mutants lacking a functional *acrB* or *tolC* do not have an altered aggregative***
185 ***ability***

186 To determine whether inactivation of AcrAB-TolC had altered the intrinsic
187 aggregative nature of the strains lacking *acrB* or *tolC* a settle assay was used, this
188 showed no significant difference in the aggregative ability of L829 (*tolC::cat*) or L830
189 (*acrB::aph*) (Figure S1A). Salt aggregation tests also showed no defect in the
190 mutants' ability to aggregate. In fact, L829 (*tolC::cat*) cells aggregated in a lower
191 concentration of ammonium sulphate than L828 (wild-type) showing a slightly greater
192 tendency for cells to aggregate than the wild-type (Figure S1B).

193 ***AcrAB-TolC does not export a factor required for biofilm formation***

194 If a soluble biofilm promoting factor was exported by AcrAB-TolC, addition of culture
195 supernatant conditioned by growth with L828 (wild-type) should be able to rescue the
196 ability of the *tolC* and *acrB* mutant strains to form a biofilm. However, two co-

197 incubation assays with wild-type and mutant strains suggested that there is no
198 'biofilm factor' exported by AcrAB-TolC. Transwell assays showed the same poor
199 ability to form a biofilm of the *acrB* and *tolC* mutants when incubated alone or co-
200 incubated with L828 (wild-type) (Figure S2). In addition, no rescue of the biofilm
201 defect was observed when co-incubated with logarithmic or stationary phase cultures
202 of L828 (wild-type) (Figure S2). Similarly, biofilm mat assays co-inoculated with an
203 equal ratio of wild-type and mutants showed that mutant cells did not comprise any
204 of the biofilm mats formed, whereas the corresponding planktonic culture comprised
205 an equal mixture of mutant and wild-type cells (data not shown).

206 ***Expression of efflux and biofilm regulator genes differs between *acrB* and *tolC****
207 ***mutants and an *acrA* mutant***

208 To explore the key observation that L1271 (*acrA::aph*) was not compromised in its
209 ability to form a biofilm we compared the expression of genes known to regulate
210 efflux gene and curli gene expression between this strain and the *acrB* and *tolC*
211 mutants.^{9, 13} This analysis revealed that *ramA* is significantly over-expressed in *acrB*
212 and *tolC* mutants but not in the *acrA* mutant.¹³ Expression of *ramA* in these strains
213 was also measured by RT-PCR and a *ramA* promoter-*gfp* fusion and results were
214 consistent in showing up-regulation of *ramA* in the *acrB* (average ~2 fold) and *tolC*
215 (average ~4 fold) mutants but not in the *acrA* mutant. The transcriptome of a *ramA*
216 over-expressing strain was also investigated to identify alterations in expression of
217 biofilm relevant genes; L786 (SL1344 pTrc::*ramA*) showed repression (two to five
218 fold) of all the curli genes, including *csgDEFG*.^{7, 11} This observation suggested that
219 the biofilm defect in the *acrB* and *tolC* mutants was mediated by repression of curli
220 biosynthesis and that this may be mediated by up-regulation of *ramA*.

221 ***The lack of curli production in mutants lacking *acrB* and *tolC* is due to***
222 ***transcriptional repression***

223 Congo red supplemented agar and Congo red staining of bacterial suspensions
224 showed qualitatively and quantitatively that curli expression is repressed in the *tolC*
225 and *acrB* mutants and produced at wild-type levels in the *acrA* mutant.⁹ This lack of
226 curli production was found to result from transcriptional repression of various genes
227 in the curli biosynthetic loci, as measured by cRT-PCR including the regulator *csgD*
228 and all the structural and assembly genes also needed to produce curli. Expression
229 of these genes was repressed in the *tolC* and *acrB* mutants but not in the *acrA*
230 mutant (Figure 3).

231 ***Role of RamA, MarA and SoxS in repression of curli production and a loss of***
232 ***biofilm formation***

233 To investigate whether RamA, MarA and SoxS are able to repress biofilm formation
234 each was over-expressed in L828 (wild-type) and the consequences investigated.
235 Artificial over-expression of *ramA* in L828 (wild-type) resulted in a complete loss of
236 the ability of the strain to form a biofilm, over-expression of *marA* and *soxS* also
237 resulted in a loss of biofilm formation although to a lesser extent than that seen with
238 *ramA* (Figure 4). Production of curli on Congo red agar was repressed in each over-
239 expression strain.

240 Spatial expression of *ramA*, *soxS* and *marA* within colonies of L828 (wild-type), L829
241 (*tolC::cat*) and L830 (*acrB::aph*) was visualised by fluorescence using regulator-*gfp*
242 reporter plasmids. Figure 5 shows the pattern of expression seen for both *ramA* and
243 *marA* was the inverse of where curli was being produced in each colony on Congo
244 red agar. In wild-type colonies the highest *ramA* and *marA* expression was seen at
245 the perimeter, where curli expression was at it's lowest. In the *tolC* and *acrB* mutant,

246 a higher level of *ramA* and *marA* expression is seen dispersed throughout the
247 colony, again inversely correlating with phenotypic curli expression.

248 ***Inactivation of the global regulators does not restore the ability of a toIC***
249 ***mutant to form a competent biofilm***

250 As all three regulators have the ability to repress biofilm formation when
251 overexpressed and both *marA* and *soxS* are up-regulated upon inactivation of *ramA*,
252 all three genes were inactivated to establish if rescue of biofilm formation would
253 occur in *toIC* and *acrB* mutants. Inactivation of each of the regulators alone in the
254 *toIC* and *acrB* mutants failed to rescue curli production and biofilm formation,
255 however loss of each of the regulator genes was followed by consequent up-
256 regulation of the others which may compensate for their inactivation (Figure 6). A
257 series of multiple mutants lacking combinations of the three regulators also failed to
258 rescue biofilm formation (Figure 7 shows the lack of rescue of the *toIC* mutant by
259 loss of *marA*, *soxS* and *ramA*).

260

261 Discussion

262 Multidrug efflux pumps have a central role in the biology of bacteria with roles in drug
263 resistance, cell division, pathogenicity and as recently described the formation of
264 biofilms.^{9,14} Here, we investigated the mechanism which explains the inability of
265 mutants lacking AcrB and TolC, constituents of the major AcrAB-TolC system of
266 *Enterobacteriaceae*, to form a competent biofilm. Mutants of *Salmonella* lacking a
267 functional *tolC* or *acrB* were unable to form biofilms under various conditions and this
268 was not related to any defect in growth, cellular hydrophobicity/aggregative ability or
269 export of a biofilm promoting substrate. Surprisingly a mutant lacking a functional
270 AcrA (but still expressing AcrB),¹⁰ was not defective in its ability to form a biofilm.
271 Loss of *acrA* has previously been shown to result in hyper-susceptibility to various
272 drugs and a decreased ability to attach to and invade epithelial cells in tissue
273 culture.¹⁰ The phenotype of an *acrA* mutant was, however, distinct from that of
274 mutants lacking *acrB*.¹⁰ One major difference between *acrA* and *acrB/tolC* mutants
275 is expression of the global regulator *ramA*,¹³ known to positively regulate expression
276 of *acrAB* and *tolC* as well as other genes. Expression of *ramA* was up-regulated in
277 both *acrB* and *tolC* mutants but not in an *acrA* mutant and analysis of microarray
278 data showed that *ramA* up-regulation was associated with decreased expression of
279 curli biosynthetic genes,¹¹ this led us to hypothesise that curli repression was the
280 reason that the efflux mutants did not form a biofilm and that *ramA* has a role in the
281 co-ordinated regulation of efflux and biofilm formation. We confirmed the absence of
282 curli production in mutants lacking *tolC* or *acrB* is due to strict repression of all the
283 curli biosynthetic genes. We also confirmed that *ramA* over-expression repressed
284 curli production and completely abolished biofilm formation. However, inactivation of
285 *ramA* in the *tolC* and *acrB* mutants did not rescue their ability to form a biofilm. To

286 determine whether loss of *ramA* expression is compensated by other transcriptional
287 activator genes, *marA*, *soxS* and *rob* expression was measured in mutant strains
288 lacking *ramRA*, *marRA* or *soxRS*.^{8,11,15-17} Loss of *ramRA* resulted in increased
289 expression of *marA* and *soxS*, both of which have some known functional overlap
290 with RamA.¹⁸⁻¹⁹ Furthermore, over-expression of each of *ramA*, *marA* and *soxS*
291 resulted in repression of curli production and biofilm formation. This suggests
292 repression of biofilm formation and curli expression may be a core role for
293 transcriptional activators that respond to stress and co-ordinate efflux up-regulation.
294 The regulation of curli expression is extremely complex with multiple pathways
295 known to impact curli production. Amongst these pathways are two component
296 systems which respond to membrane stress (*cpxRA*, *rscCB*, *envZ/ompR*), *rpoE* and
297 the lytic transglycosylases *mltC* and *mltE*.²⁰ All these systems have membrane or
298 periplasmic bound components demonstrating that curli synthesis is sensitive to
299 changes in the membrane. Whether RamA/MarA/SoxS) act directly to repress curli
300 synthesis or via one of the other currently known pathways which can influence curli
301 repression is not known. Although there is a suggested RamA/MarA/SoxS
302 consensus binding site, there is no good match for this sequence within the curli
303 locus suggesting that the action of these regulators in mediating curli repression is
304 indirect.^{7,21} Whilst we demonstrate here that over-expression of each of *ramA*, *marA*
305 and *soxS* can repress biofilm we were unable to rescue the *acrB* and *tolC* mutants
306 biofilm and curli production defect by inactivation of these genes individually or in
307 combination. Therefore, while we have shown these regulators can repress biofilm
308 formation in a manner phenotypically identical to that seen in response to loss of
309 efflux it is unclear how important they are in mediating the biofilm defect seen in
310 efflux mutants.

311 The demonstration that biofilm formation and efflux are co-ordinately but inversely
312 regulated with loss of function of multidrug efflux resulting in repression of biofilm is
313 interesting. Both de-repression of AcrAB-TolC and formation of a biofilm are in
314 themselves protective against antibiotic action, the inverse regulation observed here
315 seems counterintuitive. This relationship may act as a paradigm for other systems in
316 other species where a link between efflux and biofilm formation exists. It is possible
317 that conditions where efflux is up-regulated in response to stress represent a
318 hazardous environment where formation of a biofilm and the subsequent attachment
319 to a single site is a poor survival strategy explaining the evolution of a genetic switch
320 between the two.

321

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329 **Transparency declaration**

330 None to declare.

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405

406 **Figure legends**

407 Figure 1. Crystal violet biofilm assay quantifies biofilm formation of L828 (wild-type), L829
408 (*tolC::cat*), L830 (*acrB::aph*) and L1271 (*acrA::aph*), showing genetic inactivation of *tolC* or
409 *acrB* creates an inability to form a biofilm. Asterisks indicate significantly different average
410 values to wild-type ($p < 0.05$).

411 Figure 2. Phase contrast microscopy images of L828 (wild-type, panel A), L830 (*acrB::aph*,
412 panel B) and L829 (*tolC::cat*, panel C) at 40 X magnification after 48 hours incubation under
413 flow conditions.

414 Figure 3. Expression of curli genes. A schematic of the curli biosynthesis genes with average
415 expression values determined by RT-PCR used to colour each gene showing repression in
416 the *tolC* and *acrB* mutants. All expression values less than 50% of the wild-type were
417 statistically significantly different ($p < 0.05$).

418 Figure 4. Over-expression of *ramA*, *marA* or *soxS* represses biofilm formation. The bar chart
419 shows biofilm formation in the crystal violet assay by L828 carrying pTrc-*marA* or pTrc-*soxS*
420 or pTrc-*ramA* without or with induction with 1mM IPTG.

421 Figure 5. Expression of *ramA* and *marA* is up-regulated in efflux mutants and differentiated
422 spatially within colonies shown by *gfp* reporter plasmids and correlates with a lack of curli
423 production shown phenotypically on Congo red agar.

424 Figure 6. Inactivation of the *ram*, *mar* or *sox* loci results in compensatory up-regulation of
425 redundant regulators. The graph shows average expression data from RT-PCR in each
426 mutant and shows that when *ramRA* is inactivated in L828 (wild-type) there is a large
427 increase in expression of *marA* and *soxS* to compensate.

428 Figure 7. Crystal violet biofilm assay shows loss of *ramA*, *marA*, *soxS* and combinations
429 thereof does not rescue *tolC* mutant's biofilm defect. Asterisks indicate significantly different
430 average values to wild-type ($p < 0.05$).

431

432

433 **Table 1. List of strains used in this study.**

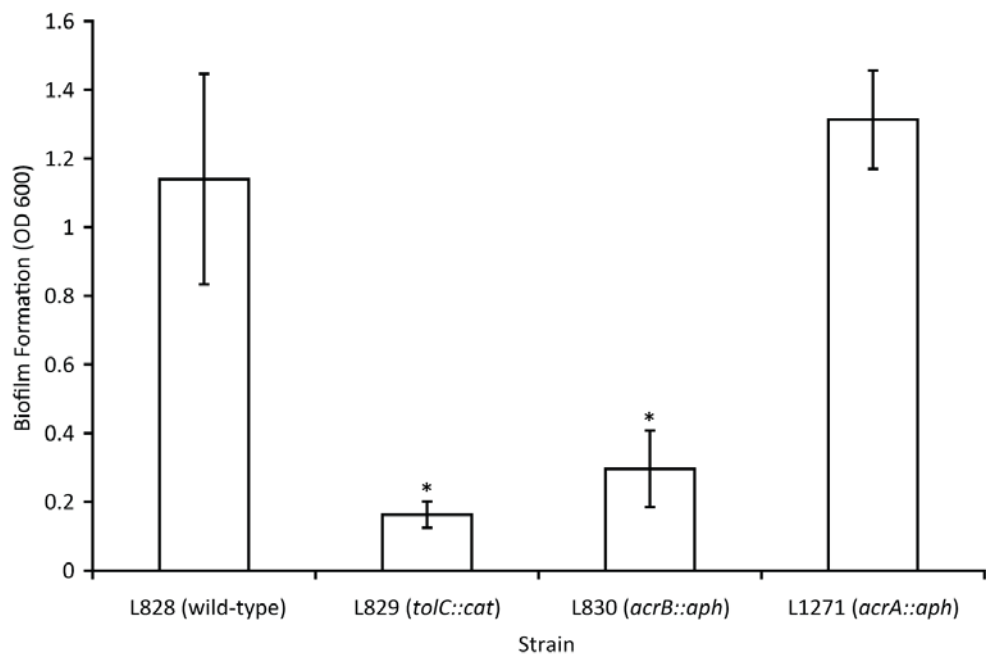
Strain	Genotype	Description	Reference
L828	14028S	Wild-type <i>Salmonella</i> Typhimurium	ATCC
L829	14028S <i>tolC::cat</i>	Mutant lacking TolC	6
L830	14028S <i>acrB::aph</i>	Mutant lacking AcrB	6
L1271	14028S <i>acrA::aph</i>	Mutant lacking AcrA	This study
L1303	14028S <i>ramRA::aph</i>	Mutant lacking RamRA	This study
L1306	14028S <i>tolC::cat, ramRA::aph</i>	Mutant lacking TolC and RamRA	This study
L1506	14028S Δ <i>tolC, \Delta</i> <i>ramA</i>	Mutant lacking TolC and RamA	This study
L1507	14028S Δ <i>tolC, \Delta</i> <i>ramA, \Delta</i> <i>soxS</i>	Mutant lacking TolC, RamA and SoxS	This study
L1508	14028S Δ <i>tolC, \Delta</i> <i>ramA, marA::aph</i>	Mutant lacking TolC, RamA and MarA	This study
L1509	14028S Δ <i>tolC, \Delta</i> <i>ramA, \Delta</i> <i>soxS, marA::aph</i>	Mutant lacking TolC, RamA, SoxS and MarA	This study
L1511	14028S <i>ramA::cat</i>	Mutant lacking RamA	This study
L1512	14028S <i>acrB::aph, ramA::cat</i>	Mutant lacking AcrB and RamA	This study
N/A	<i>E. coli</i> 042	Enterotoaggagative <i>E. coli</i>	ATCC
Plasmid			
	<i>pTrc-marA</i>	<i>marA</i> over-expression plasmid	This study
	<i>pTrc-ramA</i>	<i>ramA</i> over-expression plasmid	7
	<i>pTrc-soxS</i>	<i>soxS</i> over-expression plasmid	This study
	<i>pWKS30-marA-gfp</i>	<i>marA</i> gfp reporter plasmid	This study
	<i>pWKS30-ramA-gfp</i>	<i>ramA</i> gfp reporter plasmid	This study
	<i>pWKS30-soxS-gfp</i>	<i>soxS</i> gfp reporter plasmid	This study

Table 2. Primers used in this study.

Primer	Sequence	Description
<i>acrB</i> -checkF	GGATCACACCTTATTGCCAG	<i>acrB</i> mutant check forward primer
<i>acrB</i> -checkR	TTAACAGTGATCGTCGGTCCG	<i>acrB</i> mutant check reverse primer
<i>tolC</i> -checkF	CTTCTATCATGCCGGCGACC	<i>tolC</i> mutant check forward primer
<i>tolC</i> -checkR	CGCTTGCTGGCACTGACCTT	<i>tolC</i> mutant check reverse primer
<i>acrA</i> -checkF	ACATCCAGGATGTGTTGTCCG	<i>acrA</i> mutant check forward primer
<i>acrA</i> -checkR	CAATCGTCGGATATTGCGCT	<i>acrA</i> mutant check reverse primer
<i>pTrc-ramA</i> F	ATGACCATTTCCGCTCAGGT	<i>pTrc-ramA</i> cloning forward primer
<i>pTrc-ramA</i> R	TCAATGCGTACGGCCATGCT	<i>pTrc-ramA</i> cloning reverse primer
<i>pTrc-marA</i> F	ATGTCCAGACGCAACTGA	<i>pTrc-marA</i> cloning forward primer
<i>pTrc-marA</i> R	CTAGTAGTTGCCATGGTTCA	<i>pTrc-marA</i> cloning reverse primer
<i>pTrc-soxS</i> F	ATGTCGCATCAGCAGATAAT	<i>pTrc-soxS</i> cloning forward primer
<i>pTrc-soxS</i> R	CTACAGGCGGTGACGGTAAT	<i>pTrc-soxS</i> cloning reverse primer
<i>marA</i> -RTF	CGCAACTGACGCTATTAC	<i>marA</i> qRT-PCR forward primer
<i>marA</i> -RTR	TTCAGCGGCAGCATATAC	<i>marA</i> qRT-PCR reverse primer
<i>ramA</i> -RTF	TCCGCTCAGGTTATCGACAC	<i>ramA</i> qRT-PCR forward primer
<i>ramA</i> -RTR	AGCTTCCGTTACGCACGTA	<i>ramA</i> qRT-PCR reverse primer
<i>soxS</i> -RTF	CATATCGACCAACCGCTA	<i>soxS</i> qRT-PCR forward primer
<i>soxS</i> -RTR	CGGAATACACGCGAGAAG	<i>soxS</i> qRT-PCR reverse primer
16S-RTF	CCTCAGCACATTGACGTTAC	16S qRT-PCR forward primer
16S-RTR	TTCCTCCAGATCTCTACGCA	16S qRT-PCR reverse primer

<i>csgA</i> -RTF	AGCATTTCGCAGCAATCGTAG	<i>csgA</i> qRT-PCR forward primer
<i>csgA</i> -RTR	TTAGCGTTCCACTGGTCGAT	<i>csgA</i> qRT-PCR reverse primer
<i>csgB</i> -RTF	ATCAGGCGGCCATTATTGGT	<i>csgB</i> qRT-PCR forward primer
<i>csgB</i> -RTR	TACTGGCATCGTTGGCATTG	<i>csgB</i> qRT-PCR reverse primer
<i>csgC</i> -RTF	AATTCTCTCTGTGCGCGACG	<i>csgC</i> qRT-PCR forward primer
<i>csgC</i> -RTR	GCAGTGATTGTCCGTCCGAA	<i>csgC</i> qRT-PCR reverse primer
<i>csgD</i> -RTF	GGTATTCTGCGTGGCGAATG	<i>csgD</i> qRT-PCR forward primer
<i>csgD</i> -RTR	AGTAATGCGGACTCGGTGCT	<i>csgD</i> qRT-PCR reverse primer
<i>csgE</i> -RTF	ACGCTATCTGACCTGGATTG	<i>csgE</i> qRT-PCR forward primer
<i>csgE</i> -RTR	CGTTATGGTGATCCAGCTTC	<i>csgE</i> qRT-PCR reverse primer
<i>csgF</i> -RTF	GACGTTCCAGTTCGCTAATC	<i>csgF</i> qRT-PCR forward primer
<i>csgF</i> -RTR	ATCGTTGGTCACCATACGTC	<i>csgF</i> qRT-PCR reverse primer
<i>csgG</i> -RTF	CTGGAACGACAAGGCTTACA	<i>csgG</i> qRT-PCR forward primer
<i>csgG</i> -RTR	TGATCCAGCTGATACTGCGT	<i>csgG</i> qRT-PCR reverse primer

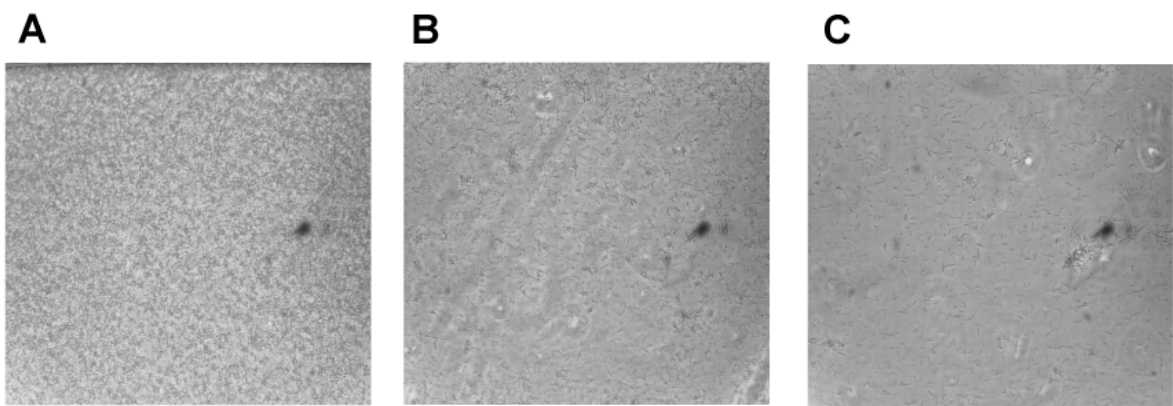
437 **Figure 1**



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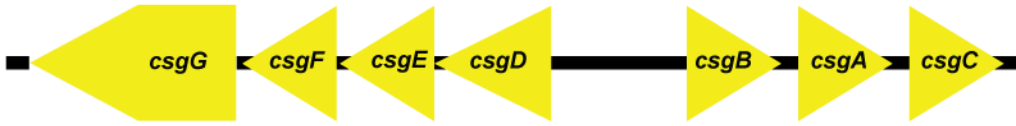
440 **Figure 2**



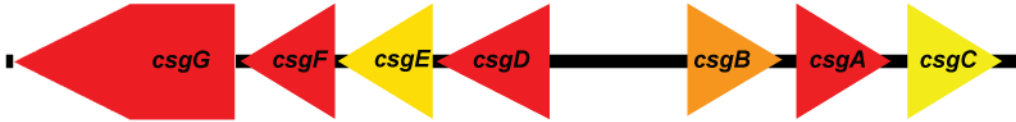
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442 **Figure 3**

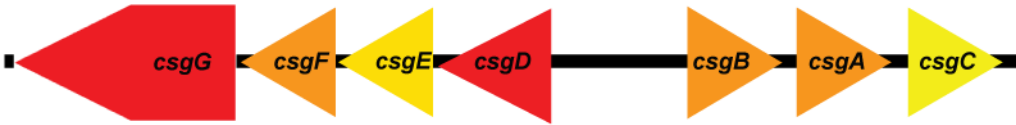
L828 (wild-type)



L829 (*tolC::aph*)



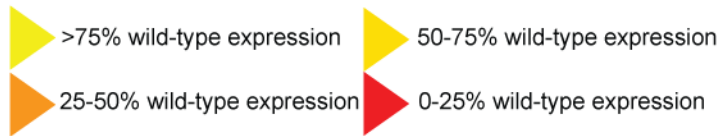
L830 (*acrB::aph*)



L1271 (*acrA::aph*)

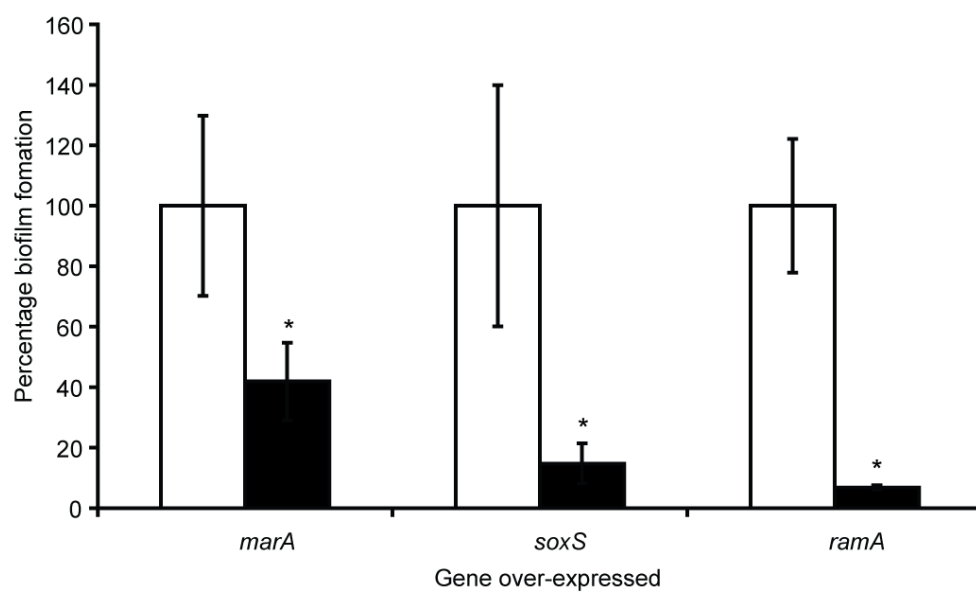


Key (Expression relative to wild-type):



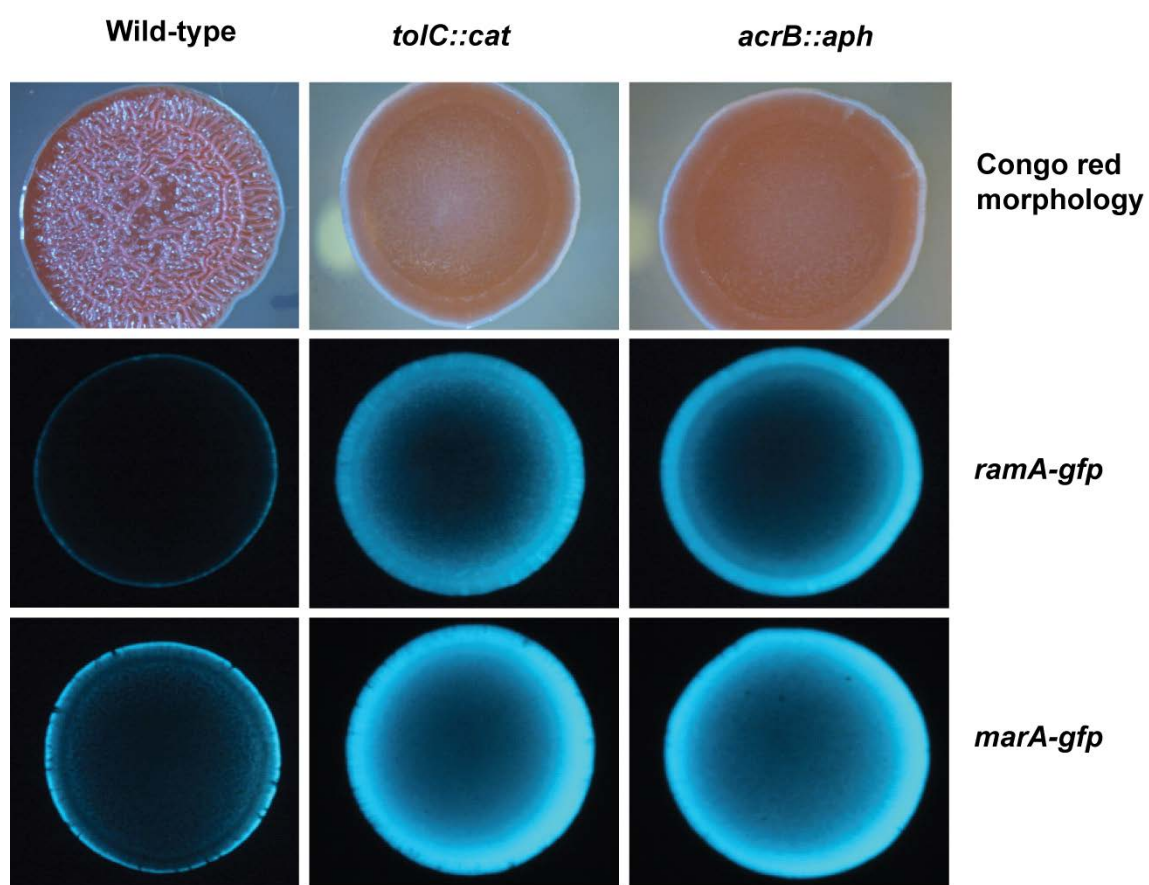
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444 **Figure 4**



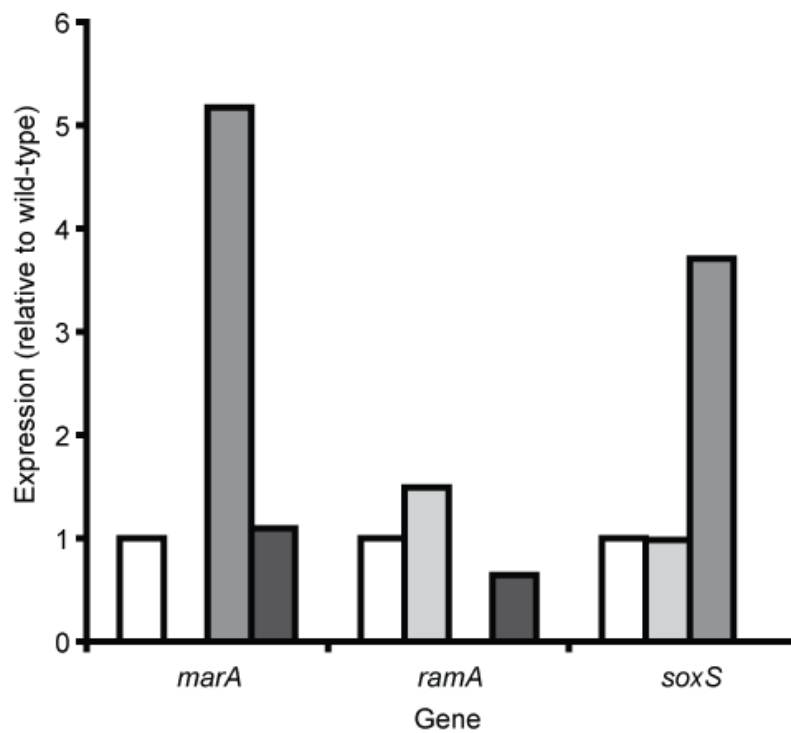
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446 **Figure 5**



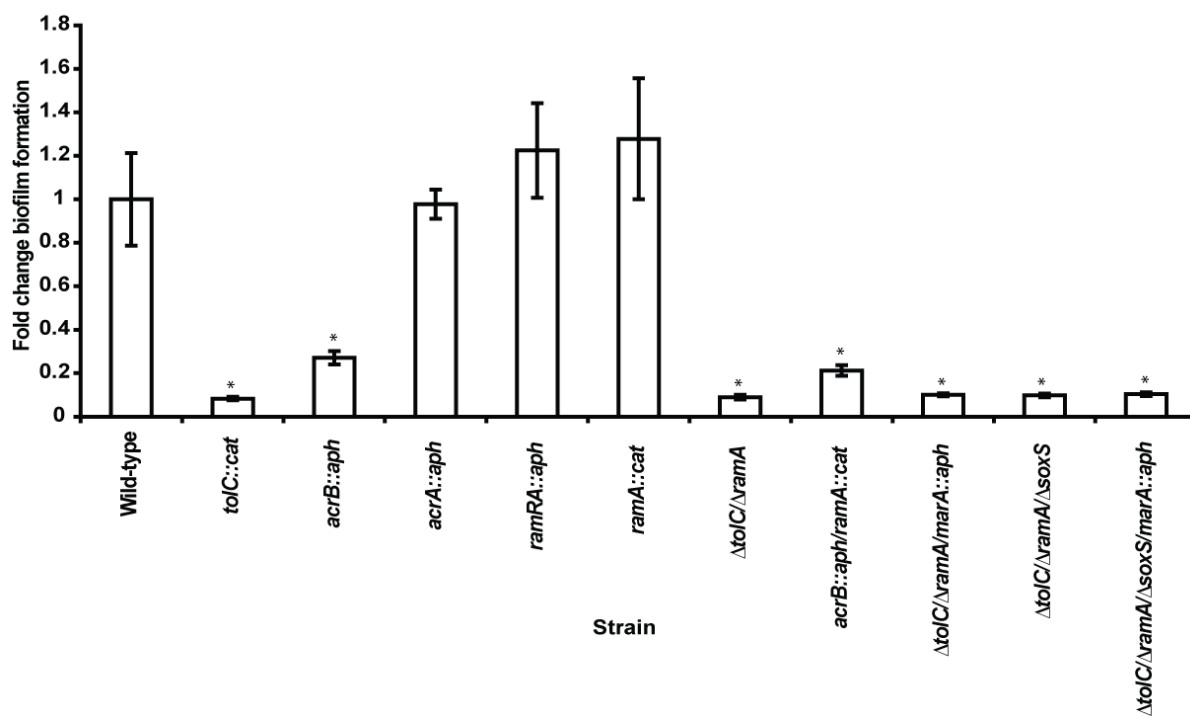
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448 **Figure 6**



449

450 **Figure 7**



451

452 **SUPPLEMENTARY FIGURES**

453 **Figure S1.**

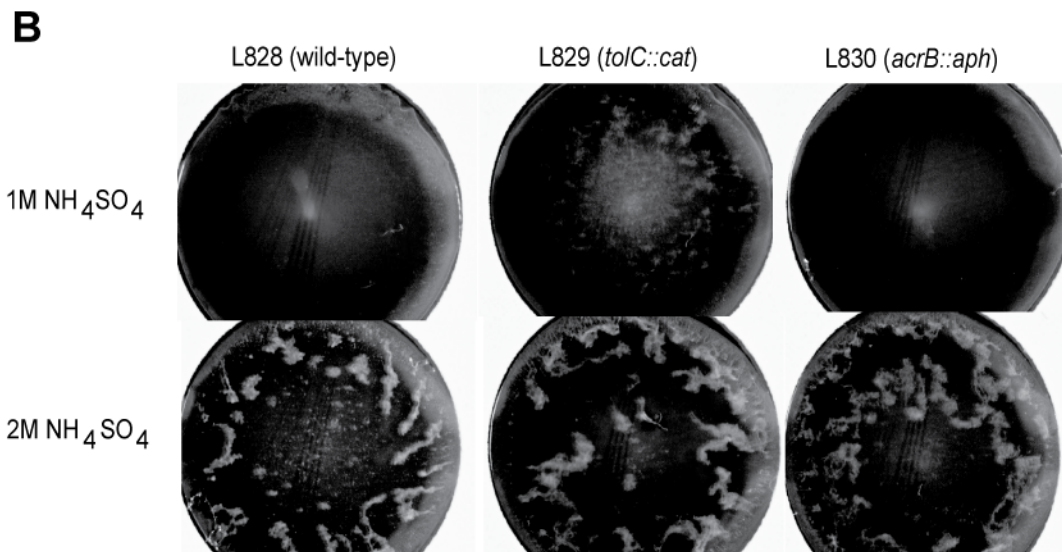
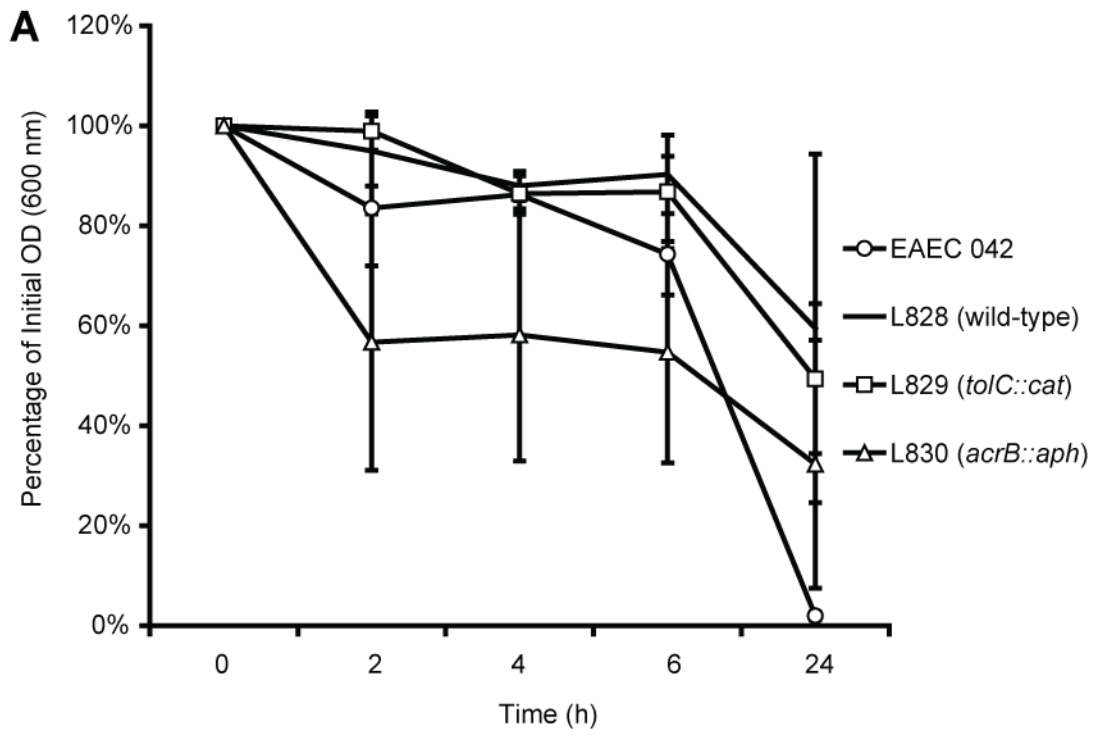
454 Panel A. Settle assay of L828 (wild-type), L829 (*tolC::cat*) and L830 (*acrB::aph*). *E. coli* O42
455 was used as a positive, aggregative control. Values indicate the percentage of an initial
456 absorbance from readings taken immediately below the surface of the liquid of a broth which
457 was incubated statically over a 24h period.

458 Panel B. Salt aggregation test images of L828 (wild-type), L829 (*tolC::cat*) and L830
459 (*acrB::aph*) in 1M and 2M ammonium sulphate. Aggregation was recorded as formation of a
460 visible precipitate and the lowest concentration of ammonium sulphate to prompt
461 precipitation recorded for each strain.

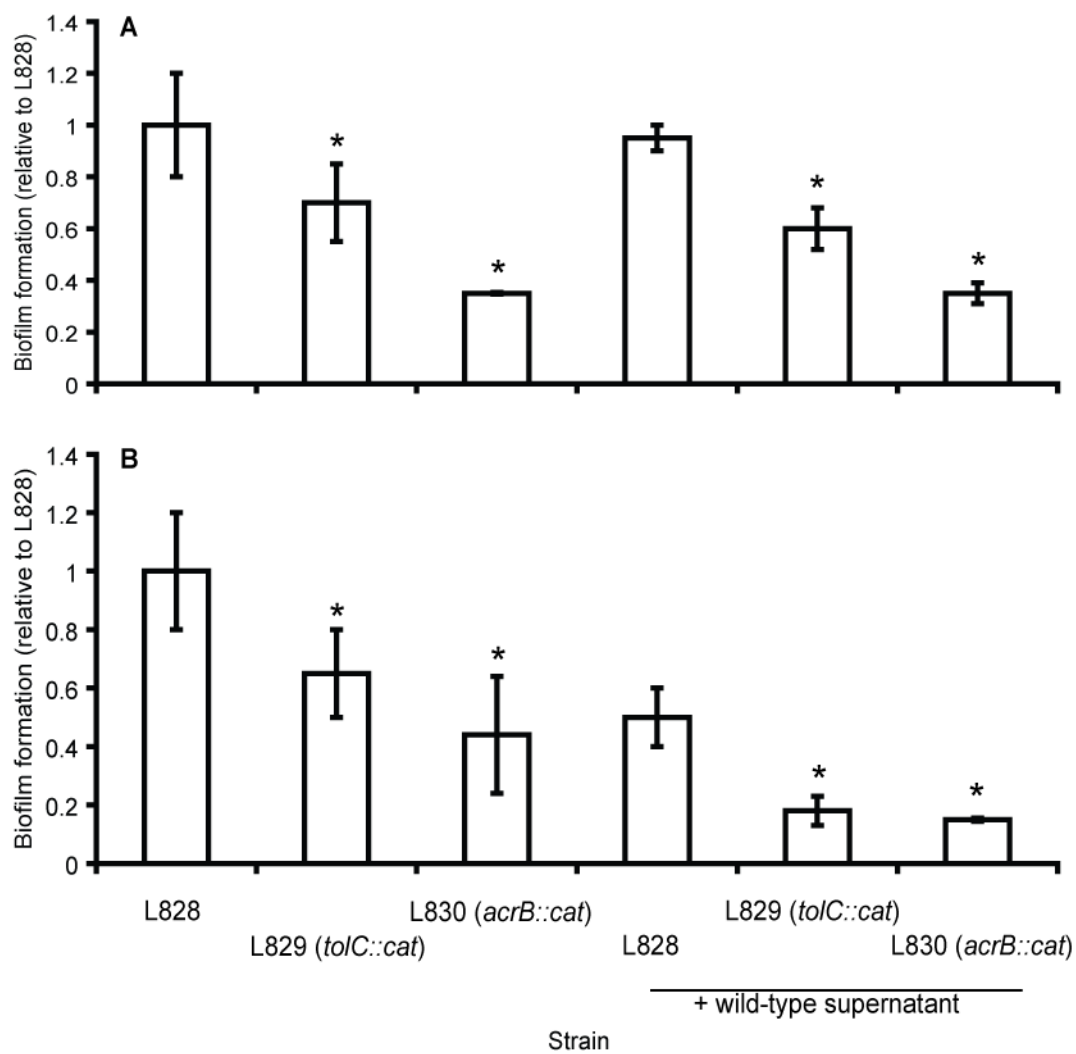
462

463 **Figure S2.** Transwell assays show no rescue of biofilm formation by mutants when co-
464 incubated with wild-type. **(A)** shows strains incubated with and without the presence of L828
465 inoculated at the same density as the mutants, **(B)** shows the same experiment but with co-
466 incubation with an overnight, undiluted culture of L828.

467



471 **Figure S2**



472

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474